

FORM PTO 1996
(REV. 5/95)

US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NUMBER
2001 0515A

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. §371

U.S. APPLICATION NO.
(of known U.S. PAT. 1.5)
NEW 097830338

International Application No.
PCT/JP99/05841

International Filing Date
October 22, 1999

Priority Date Claimed
October 26, 1998

Title of Invention

MONOCLONAL ANTIBODIES AGAINST HUMAN APOPTOSIS INHIBITORY PROTEIN NAIP AND METHOD FOR ASSAYING THE NAIP

Applicant(s) For DO/EO/US

Johe IKEDA and Harumi SAKAI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:


1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)). **ATTACHMENT A**
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19.
9. ☒ An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). **ATTACHMENT B**
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **ATTACHMENT C**
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.

☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ Other items or information:

THE COMMISSIONER IS AUTHORIZED
TO CHARGE ANY DEFICIENCY IN THE
FEE FOR THIS PAPER TO DEPOSIT
ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. 09/7830338 NEW		INTERNATIONAL APPLICATION NO. PCT/IP99/05841		ATTORNEY'S DOCKET NO. 2001 0515A	
15. [X] The following fees are submitted				CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 International Search Report has been prepared by the EPO or JPO \$ 860.00 International preliminary examination fee not paid to USPTO but international search paid to USPTO \$ 710.00 International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) \$ 690.00 International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	-20 =		X \$18.00	\$	
Independent Claims	4 - 3 =	1	X \$80.00	\$80.00	
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$270.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,210.00	
<input type="checkbox"/> Small Entity Status is hereby asserted. Above fees are reduced by 1/2.				\$	
SUBTOTAL =				\$1,210.00	
Processing fee of \$130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
TOTAL NATIONAL FEE =				\$1,210.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property				+	\$
TOTAL FEES ENCLOSED =				\$1,210.00	
				Amount to be refunded	\$
				Amount to be charged	\$
a. [X] A check in the amount of <u>\$1,210.00</u> to cover the above fees is enclosed. A duplicate copy of this form is enclosed. b. [] Please charge my Deposit Account No. 23-0975 in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. [] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>23-0975</u> .					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
19. CORRESPONDENCE ADDRESS <div style="text-align: center;">  000513 PATENT TRADEMARK OFFICE </div>			By: <u>Warren M. Cheek, Jr.</u> Warren M. Cheek, Jr., Registration No. 33,367 WENDEROTH, LIND & PONACK, L.L.P. 2033 "K" Street, N.W., Suite 800 Washington, D.C. 20006-1021 Phone: (202) 721-8200 Fax: (202) 721-8250 April 26, 2001		

[CHECK NO. 44198]

[2001_0515A]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Johe IKEDA et al.

Serial No. 09/830,338

Filed April 26, 2001



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Docket No. 2001-0515A

Group Art Unit Not Yet Assigned

Examiner Not Yet Assigned

MONOCLONAL ANTIBODIES AGAINST
HUMAN APOPTOSIS INHIBITORY PROTEIN
NAIP AND METHOD FOR ASSAYING
THE NAIP

THE COMMISSIONER IS AUTHORIZED
TO CHARGE ANY DEFICIENCY IN THE
FEE FOR THIS PAPER TO DEPOSIT
ACCOUNT NO. 23-0975.

AMENDMENT

Assistant Commissioner for Patents,
Washington, D.C. 20231

Sir:

Responsive to the Notice dated June 12, 2001, please amend the above-identified
application as follows:

In the Specification:

Page 1, immediately after the title, please insert:

This application is a 371 of PCT/JP99/05841, filed October 22, 1999.

In the Sequence Listing:

Please replace the Sequence Listing of record pages 1-11 with the attached substitute
Sequence Listing consisting of pages 1-6.

REMARKS

The foregoing amendments are presented to place the application in compliance with the
sequence rules under 37 CFR 1.821-1.825.

Applicants have submitted a revised Sequence Listing in both paper and computer readable form as required by 37 C.F.R. 1.821(c) and (e). The content of the paper and computer readable copies are the same and no new matter has been added.

In view of the foregoing, it is believed that each requirement set forth in the Notice has been satisfied, and that the application is now in compliance with the sequence rules under 37 CFR 1.821-1.825. Accordingly, favorable examination on the merits is respectfully requested.

Respectfully submitted,

Johe IKEDA et al.

By: Warren M. Cheek, Jr.
Warren M. Cheek, Jr.
Registration No. 33,367
Attorney for Applicants

WMC/gtn
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
August 13, 2001

SEQUENCE LISTING



<110> IKEDA, Johe
SAKAI, Harumi

<120> Monoclonal Antibodies Against Human Apoptosis Inhibitory Protein NAIP,
and Method For Assaying the NAIP

<130> 2001-0515A/WMC/00653

<140> 09/830,338

<141> 2001-04-26

<150> PCT/JP99/05841

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SEQUENCE LISTING

<110> Japan Science and Technology Corporation,
and Harumi SAKAI

<120> Monoclonal antibodies against human apoptosis inhibitory protein NAIP,
and method for assaying the NAIP

<130> 99-F-051PCT/YS

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09/830338 PCT/JP99/05841

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PCT09

RAW SEQUENCE LISTING

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 43 Leu Lys Ser Arg Leu Arg Gly Gly Lys Met Arg Tyr Gln Glu Glu
 44 145 150 155 160
 45 Ala Arg Leu Ala Ser Phe Arg Asn Trp Pro Phe Tyr Val Gln Gly Ile
 46 165 170 175
 47 Ser Pro Cys Val Leu Ser Glu Ala Gly Phe Val Phe Thr Gly Lys Gln
 48 180 185 190
 49 Asp Thr Val Gln Cys Phe Ser Cys Gly Gly Cys Leu Gly Asn Trp Glu
 50 195 200 205
 51 Glu Gly Asp Asp Pro Trp Lys Glu His Ala Lys Trp Phe Pro Lys Cys
 52 210 215 220

RAW SEQUENCE LISTING

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TIME: 15:41:41

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53  Glu Phe Leu Arg Ser Lys Lys Ser Ser Glu Glu Ile Thr Gln Tyr Ile
54  225                230                235                240
55  Gln Ser Tyr Lys Gly Phe Val Asp Ile Thr Gly Glu His Phe Val Asn
56                245                250                255
57  Ser Trp Val Gln Arg Glu Leu Pro Met Ala Ser Ala Tyr Cys Asn Asp
58                260                265                270
59  Ser Ile Phe Ala Tyr Glu Glu Leu Arg Leu Asp Ser Phe Lys Asp Trp
60                275                280                285
61  Pro Arg Glu Ser Ala Val Gly Val Ala Ala Leu Ala Lys Ala Gly Leu
62                290                295                300
63  Phe Tyr Thr Gly Ile Lys Asp Ile Val Gln Cys Phe Ser Cys Gly Gly
64  305                310                315                320
65  Cys Leu Glu Lys Trp Gln Glu Gly Asp Asp Pro Leu Asp Asp His Thr
66                325                330                335
67  Arg Cys Phe Pro Asn Cys Pro Phe Leu Gln Asn Met Lys Ser Ser Ala
68                340                345                350
69  Glu Val Thr Pro Asp Leu Gln Ser Arg Gly Glu Leu Cys Glu Leu Leu
70                355                360                365
71  Glu Thr Thr Ser Glu Ser Asn Leu Glu Asp Ser Ile Ala Val Gly Pro
72                370                375                380
73  Ile Val Pro Glu Met Ala Gln Gly Glu Ala Gln Trp Phe Gln Glu Ala
74  385                390                395                400
75  Lys Asn Leu Asn Glu Gln Leu Arg Ala Ala Tyr Thr Ser Ala Ser Phe
76                405                410                415
77  Arg His Met Ser Leu Leu Asp Ile Ser Ser Asp Leu Ala Thr Asp His
78                420                425                430
79  Leu Leu Gly Cys Asp Leu Ser Ile Ala Ser Lys His Ile Ser Lys Pro
80                435                440                445
81  Val Gln Glu Pro Leu Val Leu Pro Glu Val Phe Gly Asn Leu Asn Ser
82                450                455                460
83  Val Met Cys Val Glu Gly Glu Ala Gly Ser Gly Lys Thr Val Leu Leu
84  465                470                475                480
85  Lys Lys Ile Ala Phe Leu Trp Ala Ser Gly Cys Cys Pro Leu Leu Asn
86                485                490                495
87  Arg Phe Gln Leu Val Phe Tyr Leu Ser Leu Ser Ser Thr Arg Pro Asp
88                500                505                510
89  Glu Gly Leu Ala Ser Ile Ile Cys Asp Gln Leu Leu Glu Lys Glu Gly
90                515                520                525
91  Ser Val Thr Glu Met Cys Met Arg Asn Ile Ile Gln Gln Leu Lys Asn
92                530                535                540
93  Gln Val Leu Phe Leu Leu Asp Asp Tyr Lys Glu Ile Cys Ser Ile Pro
94  545                550                555                560
95  Gln Val Ile Gly Lys Leu Ile Gln Lys Asn His Leu Ser Arg Thr Cys
96                565                570                575
97  Leu Leu Ile Ala Val Arg Thr Asn Arg Ala Arg Asp Ile Arg Arg Tyr
98                580                585                590
99  Leu Glu Thr Ile Leu Glu Ile Lys Ala Phe Pro Phe Tyr Asn Thr Val
100                595                600                605
101 Cys Ile Leu Arg Lys Leu Phe Ser His Asn Met Thr Arg Leu Arg Lys

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104	625	630	635
105	Thr Pro Leu Phe Val Ala Ala Ile Cys Ala His Trp Phe Gln Tyr Pro		640
106		645	650
107	Phe Asp Pro Ser Phe Asp Asp Val Ala Val Phe Lys Ser Tyr Met Glu		655
108		660	665
109	Arg Leu Ser Leu Arg Asn Lys Ala Thr Ala Glu Ile Leu Lys Ala Thr		670
110		675	680
111	Val Ser Ser Cys Gly Glu Leu Ala Leu Lys Gly Phe Phe Ser Cys Cys		685
112		690	695
113	Phe Glu Phe Asn Asp Asp Asp Leu Ala Glu Ala Gly Val Asp Glu Asp		700
114		705	710
115	Glu Asp Leu Thr Met Cys Leu Met Ser Lys Phe Thr Ala Gln Arg Leu		715
116		725	730
117	Arg Pro Phe Tyr Arg Phe Leu Ser Pro Ala Phe Gln Glu Phe Leu Ala		735
118		740	745
119	Gly Met Arg Leu Ile Glu Leu Leu Asp Ser Asp Arg Gln Glu His Gln		750
120		755	760
121	Asp Leu Gly Leu Tyr His Leu Lys Gln Ile Asn Ser Pro Met Met Thr		765
122		770	775
123	Val Ser Ala Tyr Asn Asn Phe Leu Asn Tyr Val Ser Ser Leu Pro Ser		780
124		785	790
125	Thr Lys Ala Gly Pro Lys Ile Val Ser His Leu Leu His Leu Val Asp		795
126		805	810
127	Asn Lys Glu Ser Leu Glu Asn Ile Ser Glu Asn Asp Asp Tyr Leu Lys		815
128		820	825
129	His Gln Pro Glu Ile Ser Leu Gln Met Gln Leu Leu Arg Gly Leu Trp		830
130		835	840
131	Gln Ile Cys Pro Gln Ala Tyr Phe Ser Met Val Ser Glu His Leu Leu		845
132		850	855
133	Val Leu Ala Leu Lys Thr Ala Tyr Gln Ser Asn Thr Val Ala Ala Cys		860
134		865	870
135	Ser Pro Phe Val Leu Gln Phe Leu Gln Gly Arg Thr Leu Thr Leu Gly		875
136		885	890
137	Ala Leu Asn Leu Gln Tyr Phe Phe Asp His Pro Glu Ser Leu Ser Leu		895
138		900	905
139	Leu Arg Ser Ile His Phe Pro Ile Arg Gly Asn Lys Thr Ser Pro Arg		910
140		915	920
141	Ala His Phe Ser Val Leu Glu Thr Cys Phe Asp Lys Ser Gln Val Pro		925
142		930	935
143	Thr Ile Asp Gln Asp Tyr Ala Ser Ala Phe Glu Pro Met Asn Glu Trp		940
144		945	950
145	Glu Arg Asn Leu Ala Glu Lys Glu Asp Asn Val Lys Ser Tyr Met Asp		955
146		965	970
147	Met Gln Arg Arg Ala Ser Pro Asp Leu Ser Thr Gly Tyr Trp Lys Leu		975
148		980	985
149	Ser Pro Lys Gln Tyr Lys Ile Pro Cys Leu Glu Val Asp Val Asn Asp		990
150		995	1000
			1005

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153 Ser Ala Ser Gln Arg Ile Glu Leu His Leu Asn His Ser Arg Gly Phe
154      1025                      1030                      1035                      1040
155 Ile Glu Ser Ile Arg Pro Ala Leu Glu Leu Ser Lys Ala Ser Val Thr
156      1045                      1050                      1055
157 Lys Cys Ser Ile Ser Lys Leu Glu Leu Ser Ala Ala Glu Gln Glu Leu
158      1060                      1065                      1070
159 Leu Leu Thr Leu Pro Ser Leu Glu Ser Leu Glu Val Ser Gly Thr Ile
160      1075                      1080                      1085
161 Gln Ser Gln Asp Gln Ile Phe Pro Asn Leu Asp Lys Phe Leu Cys Leu
162      1090                      1095                      1100
163 Lys Glu Leu Ser Val Asp Leu Glu Gly Asn Ile Asn Val Phe Ser Val
164      1105                      1110                      1115                      1120
165 Ile Pro Glu Glu Phe Pro Asn Phe His His Met Glu Lys Leu Leu Ile
166      1125                      1130                      1135
167 Gln Ile Ser Ala Glu Tyr Asp Pro Ser Lys Leu Val Lys Leu Ile Gln
168      1140                      1145                      1150
169 Asn Ser Pro Asn Leu His Val Phe His Leu Lys Cys Asn Phe Phe Ser
170      1155                      1160                      1165
171 Asp Phe Gly Ser Leu Met Thr Met Leu Val Ser Cys Lys Lys Leu Thr
172      1170                      1175                      1180
173 Glu Ile Lys Phe Ser Asp Ser Phe Phe Gln Ala Val Pro Phe Val Ala
174      1185                      1190                      1195                      1200
175 Ser Leu Pro Asn Phe Ile Ser Leu Lys Ile Leu Asn Leu Glu Gly Gln
176      1205                      1210                      1215
177 Gln Phe Pro Asp Glu Glu Thr Ser Glu Lys Phe Ala Tyr Ile Leu Gly
178      1220                      1225                      1230
179 Ser Leu Ser Asn Leu Glu Glu Leu Ile Leu Pro Thr Gly Asp Gly Ile
180      1235                      1240                      1245
181 Tyr Arg Val Ala Lys Leu Ile Ile Gln Gln Cys Gln Gln Leu His Cys
182      1250                      1255                      1260
183 Leu Arg Val Leu Ser Phe Phe Lys Thr Leu Asn Asp Asp Ser Val Val
184      1265                      1270                      1275                      1280
185 Glu Ile Ala Lys Val Ala Ile Ser Gly Gly Phe Gln Lys Leu Glu Asn
186      1285                      1290                      1295
187 Leu Lys Leu Ser Ile Asn His Lys Ile Thr Glu Glu Gly Tyr Arg Asn
188      1300                      1305                      1310
189 Phe Phe Gln Ala Leu Asp Asn Met Pro Asn Leu Gln Glu Leu Asp Ile
190      1315                      1320                      1325
191 Ser Arg His Phe Thr Glu Cys Ile Lys Ala Gln Ala Thr Thr Val Lys
192      1330                      1335                      1340
193 Ser Leu Ser Gln Cys Val Leu Arg Leu Pro Arg Leu Ile Arg Leu Asn
194      1345                      1350                      1355                      1360
195 Met Leu Ser Trp Leu Leu Asp Ala Asp Asp Ile Ala Leu Leu Asn Val
196      1365                      1370                      1375
197 Met Lys Glu Arg His Pro Gln Ser Lys Tyr Leu Thr Ile Leu Gln Lys
198      1380                      1385                      1390
199 Trp Ile Leu Pro Phe Ser Pro Ile Ile Gln Lys

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200          1395          1400          1403
202 <210> SEQ ID NO: 2
203 <211> LENGTH: 5984
204 <212> TYPE: DNA
205 <213> ORGANISM: Homo sapiens
207 <220> FEATURE:
W--> 208 <221> NAME/KEY: CDC
209 <222> LOCATION: (292)..(4500)
211 <400> SEQUENCE: 2
C--> 212 acaaaagggtc ctgtgtctcac ctggggaccct tctggacgtt gccctgtgtt cctcttccgcc 60
213 tgccctgttca tctacgacga accccgggta ttgaccccag acaacaatgc cacttcoatat 120
214 tgggggacttc gtctgggatt ccaagggtgca ttcatgtcaa agttccttaa atattttctc 180
215 actgcttctct actaaaggac ggacagagca tttgttcttc agccacatac ttctcttcca 240
216 ctggccagca ttctctctca ttagactaga actgtggata aacctcagaa aatggccacc 300
217 cagcagaaag cctctgacga gaggatctcc cagtttgatc acaatttgtc gccagagctg 360
218 tctgtctcttc tgggacctaga tgcagttcac ttggcaaaag aactagaaga agaggagcag 420
219 aaggagcgag caaaaatgca gaaaggctac aactctcaaa tgcgcagtga agcaaaaagg 480
220 ttaaagacct ttactgacta tgagccgtac agctcatgga taccacagga gatggcggcc 540
221 gctgggtttt acttcaactg ggtaaaaatc gggattcacg gcttctgctg tagcctaatac 600
222 ctcttttggtg ccggcctcac gagactcccc atagaagacc acaagagggt tcatccagat 660
223 tgtgggttcc ttgtgaacaa ggtgttgggt aacattgcca agtacgacat aagggtgaag 720
224 aatctgaaga gcaggctgag agggagtaaa atgaggtacc aagaagagga ggctagactt 780
225 gcctcttcca ggaactggcc attttatgtc caagggatat ccccttgtgt gctctcagag 840
226 gctggctttg tctttacagg taaacaggac acggtacagt gttttccgtg tgggtgatgt 900
227 ttaggaaatt ggggaagaag agatgatcct tgggaagAAC atgccaaatg gttccccaaa 960
228 tgtgaatttc ttcgagtaga gaaatctcca gaggaaatTA cccagtatat tcaaaagtac 1020
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233 aaatggcagg aagggtgatga cccattagac gatcacacca gatgttttcc caattgtcca 1320
234 ttctccaaaa atatgaagtc ctctgcggaa gtgactccag accttcagag ccgtggtgaa 1380
235 ctttgtgaat tactggaAAC cacaagtgaA agcaatottg aagattcaat agcagtttgt 1440
236 cctatagtgc cagaaatggc acagggtgaa gccagtggtt ttcaagaggc aaagaatctg 1500
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238 atctcttccg actctggccac ggaccacttg ctgggctgtg atctgtctat tctgttcaaa 1620
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242 ctctccctta gttccaccag accagacgag gggctggcca gtatcactct tgaccagctc 1860
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244 aaacaggctc tattctcttt agtgaactac aaagaatatat gttcaatccc tcaagtcata 1980
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250 gtggcgtgtt tcaagtctca tatggaaagg ctttctctaa ggacaaaagg gacagctgaa 2340
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VERIFICATION SUMMARY

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PATENT APPLICATION: US/09/830,338

TIME: 15:41:42

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Output Set: N:\CRF3\03072002\I830338.raw

L:12 M:271 C: Current Filing Date differs, Replaced Current Filing Date
L:208 M:257 W: Feature value mis-spelled or invalid, <221> Name/Key for SEQ ID#:2
L:212 M:112 C: (48) String data converted to lower case,
M:112 Repeated in SeqNo=2

21PA¹⁵

09/830338

JC03 Rec'd PCT/PTO 26 APR 2001

1

DESCRIPTION

Monoclonal Antibodies against Human Apoptosis Inhibitory Protein NAIP
and Method for Assaying The NAIP

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Technical Field

The present invention relates to monoclonal antibodies, which
specifically recognize Human Apoptosis Inhibitory Protein NAIP and an
immunoassay method of the NAIP.

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Background Art

Apoptosis is a type of programmed death of a cell, in which
phenomena such as lack of contact with the surrounding cells, inspissation
of cytoplasm, aggregation of chromatin and karyopyknosis related to the
activity of endonuclease, fragmentation of nucleus, the cell being changed
into membrane-wrapped bulboid corpuscles, englobement of the bulboid
corpuscles by the adjacent macrophage or epithelial cells, or fragmentation of
the nucleosome unit of DNA into DNA fragments of 180-200 base length by
the activity of endonuclease are observed. Apoptosis has been discussed as
a mechanism in which the final fragments of apoptic somatic cells exhibiting
the aforementioned phenomena are englobed by the adjacent cells (e.g.,
"Immunology Today", 7:115-119. 1986: Science 245:301-305. 1989).

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As the gene that controls the apoptosis described above, the bcl-2
gene, which is one of oncogene discovered from B cell lymphoma in 1985, is
known. This bcl-2 gene appears quite frequently in cells of the immune
system or neuronal cells. It is assumed that the substance produced as a
result of expression of the gene suppresses the apoptosis of such cells,

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whereby the function of the human immune and neuronal systems are constantly maintained the homeostasis thereof. In addition, as the bcl-2 gene appears in a fetus in an especially wide range, it is assumed that the gene plays an important role in morphogeny during the ontogenic process.

On the other hand, the inventors of the present application have isolated Neuronal Apoptosis Inhibitory Protein (NAIP) gene, as the gene causing Spinal Muscular Atrophy (SMA) which is a familial genetic disease, from the human chromosome 5q13.1 domain (Roy et al., Cell 80: 167-178, 1995), and they have filed a patent application for the gene (PCT/CA95/00581). Specifically, it is considered that the mutation of the NAIP gene or the decrease in the number of copies therefrom causes apoptosis of spinal neurons, resulting in the development of SMA. Further, in a case in which the NAIP gene is introduced into various cultured cells and stimulation is provided to the cells to induce apoptosis, it has been found out that the death of the cells is significantly prevented (Liston et al., "Nature" 379: 349-353, 1996). In this case, it has also been found out that NAIP functions as the apoptosis inhibitory factor not only to the neuronal cells but also to the somatic cells as a whole.

The inventors of the present application have isolated the full amino acid sequence of NAIP and cDNA encoding the NAIP, and filed a patent application thereof (Japanese Patent Application No. 9-280831).

As described above, NAIP is a protein which is concerned with various apoptosis-related diseases including SMA. In order to understand the mechanism of a patient's developing such diseases, diagnose the risk for developing the diseases, prevent the development of the diseases or reduce the severity of the diseases, and develop the medical technique and medicines for treatment, it is essential to accurately assay the amount of expressed NAIP.

The inventions of the present application has been contrived in consideration of the aforementioned task, and objects of the present invention is to provide anti-NAIP monoclonal antibodies, which are essential for assaying NAIP and a NAIP assaying method using the monoclonal antibodies.

Disclosure of Invention

The inventors of the present application have assiduously studied the solution of the aforementioned task, and as a result, discovered that the epitopes of NAIP exist in the amino acids of the 256-586th and the 841-1052nd in SEQ ID NO: 1.

The present application, on the basis of the discovery, provides an anti-NAIP monoclonal antibody recognizing a human apoptosis inhibitory protein NAIP having the amino acid sequence of SEQ ID NO: 1, which is produced from hybridoma prepared by fusing a myeloma cell line with antibody-producing cell of mammal immunized by antigen containing a polypeptide which comprises amino acid sequence of the 256-586th, the 841-1052nd or parts thereof in SEQ ID NO:1

The present application provides, as specific embodiments of the monoclonal antibody: anti-NAIP monoclonal antibody hnm365, which is produced from hybridoma 656-1 (FERM BP-6919), and its epitope is the 354-365th region in SEQ ID NO: 1.; anti-NAIP monoclonal antibody hnm381, which is produced from hybridoma 656-2 (FERM BP-6920), and its epitope is the 373-387th region in SEQ ID NO: 1; and anti-NAIP monoclonal antibody hnm841, which is produced from hybridoma 841 (FERM BP-6921), and its epitope is the 841-1052nd region in SEQ ID NO:1.

The present application provides the first method of assaying NAIP,

which comprises contacting a marker-labeled anti-NAIP monoclonal antibody with a sample containing NAIP thereby binding the marker-labeled antibody with NAIP, and measuring signal strength of the marker in the bound structure.

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In the first assay method, it is preferred that the anti-NAIP monoclonal antibody is any one of said hnmnc365, hmnc381 and hmnc841, and that the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

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The present application provides the second method of assaying NAIP which comprises contacting an anti-NAIP primary antibody with a sample containing NAIP thereby binding the primary antibody with NAIP, further binding the bound structure with an anti-NAIP secondary antibody, and measuring signal strength of a marker bound with the secondary antibody, wherein:

- (1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;
- (2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or
- (3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

In the second assay method, it is preferred that the primary antibody is immobilized on solid phase, that the anti-NAIP monoclonal antibody is any one of said hnmnc365, hmnc381 and hmnc841, and that the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

25

The present application provides the first kit for assaying NAIP at least including:

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- (a) a plate on which an anti-NAIP primary antibody is immobilized; and
- (b) an anti-NAIP secondary antibody labeled with a marker, wherein:

(1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;

(2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or

5 (3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

In the first assay kit, it is preferred that the marker is a radioactive isotope, a fluorescent colorant or an enzyme, and in the case of the marker
10 being emzyme the kit further includes:

(c) a substrate which develops a color by the enzyme activity.

The present invention provides the second kit for assaying NAIP at least including:

15 (a) a plate on which an anti-NAIP primary antibody is immobilized;

(b) an anti-NAIP secondary antibody; and

(c) a marker to be bound with the secondary antibody, wherein:

(1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;

20 (2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or

(3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

25 In the second assay kit, it is preferred that the marker is a radioactive isotope, a fluorescent colorant or an enzyme, and in the case of the marker being emzyme the kit further includes:

(c) a substrate which develops a color by the enzyme activity.

30 In said assay kits, it is further preferred that the anti-NAIP monoclonal antibody is any one of said hnmnc365, hnmnc381 and hnmnc841

Brief Description of Drawings

Fig. 1 is a graph that shows a relationship between the concentration of purified NAIP in a sample solution and the absorbance measured by the method described in Examples.

Fig. 2 shows the results of Western Blotting in which anti-NAIP antibodies of a mononuclear cell solution derived from human peripheral blood were used. Lanes represent, in order, 1. monoclonal antibody hnm365; 2. monoclonal antibody hnm381; 3. monoclonal antibody hnm3841; and 4. polyclonal antibody. The concentrations of the antibodies each resulted from dilution by 250 times.

Best Mode for Carrying Out the Invention

The anti-NAIP monoclonal antibodies of the present invention may be produced by the following steps, for example, according to the known method ("Monoclonal Antibody" Takaaki NAGAMUNE and Hiroshi TERADA, Hirokawa Shoten, 1990; "Monoclonal Antibody" James W. Goding, third edition, Academic Press, 1996).

1. Preparation of hybridomas

A mammal animal is immunized by using an immunogen containing a polypeptide, the polypeptide comprising amino acid sequence of the 256-586th, the 841-1052nd or parts thereof in SEQ ID NO: 1. An additional immunization is optionally carried out according to necessity so that the animal is sufficiently immunized. Next, the antibody-producing cells (lymphatic cells or spleen cells) are isolated from the animals and fused cells are obtained by fusing the antibody-producing cells and myeloma cells. A plurality of cells that respectively produce the targeted monoclonal antibody

are selected and cultured, thereby obtaining hybridomas. The steps for the procedure will be each described in detail hereinafter.

a) Preparation of immunogen

The polypeptide having the amino acid sequence of the 265-586th in SEQ ID NO: 1 may be prepared by, for example, cleaving NAIP cDNA having the nucleotide sequence of SEQ ID NO: 2 with a restriction enzyme to obtain a DNA fragment containing the nucleotide sequence of the 1056-2049th, and expressing the DNA fragment in an appropriate host-vector system. The polypeptide having the amino acid sequence of the 841-1052th in SEQ ID NO: 1 may be prepared by expressing a DNA fragment having the nucleotide sequence of the 2812-3447th in SEQ ID NO: 2 in an appropriate host-vector system.

Alternatively, polypeptide having a partial sequence (10-20 amino acids) of the amino acids sequence of the 256-586th or the 841-1052nd region in SEQ ID NO: 1 may be prepared. In this case, by using polypeptides of different sequences, hybridomas each producing monoclonal antibody of different epitope can be obtained.

These polypeptides may be also used in a form of a fusion protein in which the polypeptide is fused with other proteins (e.g., glutation-S-transferase: GST). Use of such fusion proteins is especially preferable in terms of facilitating and ensuring the separation process of the targeted protein from the expressed product of the host-vector system and the screening process (described below) of the hybridoma.

It should be noted that the polypeptide may be that having amino acid sequence in which at least one amino acid residue is deleted or substituted or added in amino acid sequence of the 256-586 or a part in SEQ ID NO: 1.

b) Immunization of animals

As the animals to be immunized, mammals used in the known hybridoma preparation methods can be employed. Specific examples of the

animals include mice, rats, goats, sheep, cows and horses. However, in terms of availability of myeloma cells to be fused with the isolated tibody-producing cells, it is preferable to use mice or rats as the animals to be immunized. There is no particular restriction on the strains of mice and rats actually used. In the case of mice, examples of strains thereof which can be used include A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BR, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, RIII, SJL, SWR, WB, 129. In the case of rats, examples of strains thereof that can be used include Low, Lewis, Sprague, Daweley, ACI, BN, Fisher. Among them, if the suitability in being fused with the myeloma cells described below is considered, the "BALB/c" strain of mice and the "Low" strain of rats are especially preferable as the animals to be immunized. It is preferable that the mouse or rat is 5-12 week old when it is immunized.

The immunization of the animal can be carried out by subcutaneously or intraperitoneally dosing the polypeptide solution as an immunogen, into the animal. The dosing schedule of the antigen varies depending on the types of the subject animal or the differences between the individual animals. In general, the antigen is preferably dosed totally 2-6 times with 1-2 weeks of the interval between doses. The amount of the antigen to be dosed also varies depending on the types of the animal and the differences between the individual animals. In general, the amount of the antigen to be dosed is approximately 10-100 $\mu\text{g}/\mu\text{l}$.

c) Fusion of cells

1-5 days after the final immunization in the aforementioned dosing schedule, spleen cells or lymphatic cells containing the antibody-producing cells are sterilely collected from the immunized animal. The separation of the antibody-producing cells from the spleen cells or the lymphatic cells can be carried out according to the known methods.

Next, the antibody-producing cells are fused with myeloma cells. There is no particular restriction on the myeloma cells to be used, and those appropriately selected from the known cell lines may be used. However, in

consideration of the convenience at the time of selecting hybridomas from the fused cells, it is preferable to employ a HGPRT (Hypoxanthine-guanine phosphoribosyl transferase) defective line for which a selection procedure has been established. Specific examples thereof include: X63-Ag8(X63), NS1-Ag4/1(NS-1), P3X63-Ag8.UI(P3UI), X63-Ag8.653(X63.653), SP2/0-Ag14(SP2/0), MPC11-45.6TG1.7(45.6TG), FO, S149/5XXO.BU.1, which are derived from mice; 210.RSY3.Ag.1.2.3(Y3) derived from rats; and U266AR(SKO-007), GM1500 · GTG-A12(GM1500), UC729-6, LICR-LOW-HMy2(HMy2), 8226AR/NIP4-1(NP41), which are derived from human.

The antibody-producing cells may be fused with the myeloma cells in an appropriate manner, according to the known method, under a condition in which the survival rate of the cells does not drop to such an extremely low level. Examples of such methods include a chemical method in which the antigen-producing cells are mixed with the myeloma cells in a polymer (e.g., polyethylene glycol) solution of a high concentration, a physical method in which electric stimulation is utilized, and the like.

The selection of the fused cells from the non-fused cells is preferably carried out according to the known HAT (Hypoxanthine/ Aminopterin/ Thymidine) selection method. This method is effective when fused cells are obtained by using myeloma cells of a HGPRT defective line that is not viable under the presence of aminopterin. That is, by cultivating fused cells and cells which have not been fused in a HAT culture, only the fused cells that is resistant to aminopterin are selectively remained and allowed to reproduce.

d) Screening of hybridoma

The screening of the hybridoma which produce the targeted monoclonal antibody can be performed by the known EIA (Enzyme Immunoassay), RIA (Radio Immunoassay), fluorescent antibody methods and the like. When a fused protein is employed as the immunogen, the hybridoma can be screened more reliably by carrying out the aforementioned screening methods for the protein which is the partner of the fusion, as well.

By conducting such a screening process, hybridomas respectively

producing monoclonal antibodies having different epitope domains are obtained. Accordingly, the monoclonal antibodies of the present invention include all of the plural types of monoclonal antibodies respectively produced by the hybridomas prepared by the method described above.

5 After the screening process, the hybridomas are then subjected to cloning by the known methods such as the methylcellulose method, the soft agarose method and the limiting dilution method, so that the hybridomas can be used for producing the antibodies.

10 The hybridomas obtained by the aforementioned method can be stored in the frozen state in liquid nitrogen or in a freezer in which the temperature is no higher than -80°C .

2. Production of the monoclonal antibodies and purification thereof

15 The monoclonal antibodies that specifically recognize NAIP can be obtained by cultivating, according to the known method, the hybridomas prepared as described in the paragraph 1 above.

20 The cultivation may be conducted, for example, in the culture having the same composition as that used in the cloning method described above. Alternatively, in order to produce a large amount of the monoclonal antibodies, it is acceptable to inject the hybridoma intraperitoneally to a mouse and collect the monoclonal antibody from the ascites of the animal.

25 The monoclonal antibody obtained in such a manner can be purified by the methods including the ammonium sulfate salting out method, the gel filtration method, the ion-exchange chromatography method, the affinity chromatography method and the like.

Next, the NAIP assay method of the present invention will be described hereinafter.

30 In the first assay method, a solution of the marker-labeled anti-NAIP monoclonal antibody (M-mAb) is contacted with a sample containing NAIP so

that the marker-labeled monoclonal antibody is bound with NAIP, and the bound structure (M-mAb: NAIP) are separated. As the means for separation, any known methods including the chromatography method, the salting out method, the alcohol precipitation method, the enzyme method, the solid phase method and the like may be employed. In a case in which an enzyme is used as the marker, a substrate that develops a color as a result of decomposition by the enzyme activity is added. In this case, the activity of the enzyme is measured by optically measuring the amount of the decomposed substrate and the activity of the enzyme is converted into the amount of bound antibody, so that the amount of NAIP is calculated on the basis of the comparison of the obtained amount of bound antibody with the reference value. In a case in which a radioactive isotope is used as the marker, the amount of the radioactive rays emitted from the radioactive isotope is measured by a scintillation counter or the like. In a case in which a fluorescent colorant is used as the marker, a device in which a fluorescent microscope is incorporated can measure the magnitude of fluorescence.

In the second assay method, two types of antibodies whose epitope domains for NAIP are different from each other (the primary antibody and the secondary antibody) are used. Specifically, at first the primary antibody (Ab I) is contacted with a sample containing NAIP so that the primary antibody and NAIP are bound with each other. The bound structure (Ab I: NAIP) is bound with the secondary antibody that has been marker-labeled (M-Ab II), and the signal strength of the marker in the bound structure of the three components (Ab I: NAIP: M-Ab II) is measured. Optionally, in order to make the signal stronger, it is acceptable to allow the bound structure (Ab I: NAIP) to be bound, at first, with secondary antibody that is not marker-labeled and then allow the secondary antibody to be bound with the marker. Such bonding of the secondary antibody with a marker-labeled molecular can be effected, for example, by using the secondary antibody with biotin and the marker with avidin. Further, it is also acceptable that an antibody (the tertiary antibody) that recognizes a portion of the secondary antibody (e.g., Fc

domain) is marker-labeled, so that the tertiary antibody is bound with the secondary antibody (II). The anti-NAIP monoclonal antibodies of the present invention may be used for both the primary antibody and the secondary antibody. Alternatively, the anti-NAIP polyclonal antibody (the anti-serum of the animals immunized by the aforementioned polypeptide, for example) may be used for one of the primary antibody and the secondary antibody.

Although this second method can be carried out either in the liquid phase or on the solid phase, it is preferable to carry out the method on the solid phase, in order to make the assay of extremely small amounts and the operation as a whole easier. More specifically, the solid phase method includes the steps of: providing the primary antibody on a resin plate or the like so that the primary antibody is immobilized; allowing the antibody on the solid state to be bound with NAIP; washing off the NAIP which is not bound to the antibody; allowing the bound NAIP remaining on the plate to be bound with the secondary antibody; and measuring the signal strength of the secondary antibody. This is what is called the "sandwich method", and widely used as "ELISA" (enzyme linked immunospecific assay) when an enzyme is used as the marker.

In the methods described above, there is no particular limitation on the enzyme used as the marker, as long as the turn over number of the enzyme is relatively large, the enzyme is stable after being bound with the antibody, the enzyme specifically acts on the substrate so that the substrate develops a color, and other required conditions are satisfied. Examples of the enzyme include the enzymes commonly used for EIA, such as peroxidase, β -galactosidase, alkali-phosphatase, glucoseoxydase, acetylcholine-esterase, glucose-6-phosphorylation dehydrogenase, malic acid dehydrogenase and the like. Further, enzyme inhibitors and coenzymes may also be used. Bonding of these enzymes with the monoclonal antibody can be carried out according to the known method which employs a cross-linking agent such as maleimide compounds. As the substrate, any suitable known compounds may be used,

depending on the types of the enzyme that is actually used. In a case in which peroxidase is used as the enzyme, 3, 3' 5, 5'-tetramethylbenzidine may be used as the substrate. In a case in which alkali-phosphatase is used as the enzyme, para-nitrophenol or the like may be used as the substrate.

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In a case in which a radioactive isotope is used as the marker, examples of the radioactive isotope include those used in the standard RIA process such as ^{125}I and ^3H . Examples of the fluorescent colorants include those used in the standard fluorescent antibody method such as fluorescence isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC).

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The assay kits of the present invention may be used for the "sandwich method" in which the aforementioned second assay method is carried out on the solid phase. Such kits of various types are commercially available in accordance with the types of the components to be assayed. The assay kits of the present invention may be basically constituted of various components used in known and commercially available kits, except that the aforementioned anti-NAIP monoclonal antibody and/or the anti-NAIP polyclonal antibody is used as the antibodies. In addition, the assay kits of the present invention including the components described above may be provided with a washing solution for washing off the NAIP which has not been bound and/or the secondary antibody which has not been bound.

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Examples

The present invention will be described in detail by examples hereinafter. It should be noted, however, that the present invention is not limited to any of these examples.

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Example 1: Production of the monoclonal antibodies

(1) Preparation of the immunogen

The 1056-2049th region of NAIP cDNA of which nucleotide sequence is shown in SEQ ID NO: 2 was amplified, and the DNA fragment (NAIP.256-586) was inserted at the EcoR I site of pGEX-3X (by Pharmacia Co.). After confirming the nucleotide sequence, the host *Escherichia coli* BL21 (DE3) pLysS was transformed by this recombinant vector pGEX-3X(NAIP.256-586) and cultivated in the LB medium for 5 hours at 30°C. Thereafter, IPTG was added to the medium and the cultivation was continued at 20°C for 3 hours. The bacteria was separated by centrifuging, dissolved into the dissolving solution (PBS, Triton X-100), frozen at -80°C and melted, and then subjected to ultrasonic destruction. The product was centrifuged at 1000 × g for 30 minutes, the supernatant was introduced to a glutathione sepharose 4B column so as to pass through it, whereby fusion protein GST-NAIP(256-586) was obtained.

In addition, the 2812-3447th region of NAIP cDNA of which nucleotide sequence is shown in SEQ ID NO: 2 was amplified, and the DNA fragment (NAIP841-1052) was inserted at the BamHI-Sall site of pGEX-4X-3 (by Pharmacia Co.). Thereafter, the same method as described above was repeated, thereby obtaining the fusion protein GST-NAIP(841-1052).

(2) Immunization of the animal

50 µg/µl of each of the fusion proteins obtained in the aforementioned (1) was dosed to a Balb/c mouse, intraperitoneally, as the initial immunization. The second immunization was performed 2 weeks after the initial immunization, and immunization was conducted totally six times with one-week interval. At the initial immunization, the fusion protein was dosed in a state in which Freund complete adjuvant of the equal amount was mixed thereto. At the second to fifth immunization, the fusion protein was dosed in a state in which Freund incomplete adjuvant was mixed thereto. At the final immunization, only the fusion protein solution was dosed.

(3) Fusion of cells

The spleen cells were sterilely isolated three days after the final immunization. The collected spleen cells and the myeloma cell line SP2/O-Ag14 derived from mice were mixed and then subjected to the fusing treatment by using polyethylene glycol #4000. The obtained cells were planted on a 96-hole plate, and the fused cells were selected by the HAT culture.

(4) Screening

An ELISA plate on which the NAIP polypeptide used as the immunogen was provided on the solid state and an ELISA plate on which GST was provided in the solid state were prepared. Clones that did not react to the GST plate but reacted only to the NAIP plate were selected and subjected to screening. Next, among the supernatants of the cultures of respective hybridomas, the wells reacted to the NAIP polypeptide were regarded as positive. The cloning of the hybridomas was carried out by using the positive wells in the limiting dilution method. The screening process was repeated for the cultures of the hybridomas that were supposed to have only single-type clones, whereby a plurality of hybridomas was obtained. Among these plural hybridomas, hybridomas 656-1, 656-2 and hnm841 were deposited to National Institute of Bioscience and Human-Technology. The deposit Nos. of these hybridomas are FERM BP-6919 (hybridoma 656-1), FERM BP-6920 (hybridoma 656-2) and FERM BP-6921 (hybridoma hnm841), respectively.

(5) Production of the monoclonal antibodies

Three types of the hybridomas obtained as described above were dosed to a Balb/c mice, intraperitoneally, and the ascites containing the monoclonal antibody was collected after one week. From the collected ascites, the three types of monoclonal antibodies hnm365, hnm381 and hnm841 were purified by using an affinity column in which protein G was used.

It was confirmed that the monoclonal antibody hnmc365, produced by hybridoma 656-1 which had been prepared by using fusion protein GST-NAIP(256-586) as the immunogen, belongs to the subclass IgG1 and the epitope thereof is the amino acid sequence of the 254-368th region in SEQ ID NO: 1. It was also confirmed that the monoclonal antibody hnmc381 produced by hybridoma 656-2 belongs to the subclass IgG2b and the epitope thereof is the amino acid sequence of the 373-387th region in SEQ ID NO: 1. Further, it was confirmed that the monoclonal antibody hnmc841, produced by the hybridoma hnmc841 which had been prepared by using fusion protein GST-NAIP(841-1052) as the immunogen, belongs to the subclass IgG1 and the epitope thereof is the amino acid sequence of the 841-1052nd region in SEQ ID NO: 1.

Example 2: Production of the polyclonal antibody

A rabbit (Japanese White Rabbit) was immunized by the standard method, by using as the immunogen the fusion protein GST-NAIP(256-586) prepared in a manner similar to that of Example 1 (1). The anti-serum was then separated, and the polyclonal antibody was purified by a sepharose 4B column in which the aforementioned fused proteins were bonded.

Example 3: Production of ELISA kit

(1) Primary antibody-immobilized plate

A solution (20 µg/ml) of the anti-NAIP monoclonal antibody hnmc365 produced in Example 1 was dissolved into 10 mmol/l of potassium phosphate buffer (pH 7.5) containing 150 mmol/l of sodium chloride and 1 g/l of sodium azide. 50 µl of this solution was pipetted into each hole of a 96-hole plate for ELISA. The plate was stored at 4°C for 16 hours. Thereafter, the plate was washed with 10 mmol/l potassium phosphate buffer (pH 7.5) containing 150

mmol/l sodium chloride, whereby the plate on which the anti-NAIP monoclonal antibody was immobilized was produced.

(2) Biotinylated secondary antibody

5 0.01 mmol of biotin-amidecaproic acid N-hydroxysuccinic imide ester dissolved into N, N-dimethylformamide was added to 10 mg of the anti-NAIP polyclonal antibody produced in Example 2. The mixture was stored at 25°C for 3 hours and then subjected to dialysis for 16 hours in 50 mmol/l potassium phosphate buffer (pH 7.4), whereby the biotinylated anti-NAIP
10 polyclonal antibody was produced.

(3) Marker to be bound to the secondary antibody

 A solution of horse radish peroxydase-labeled streptoavidin was diluted to the concentration of 0.5 µg/ml with 10 mmol/l potassium
15 phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride and 1 g/L casein, whereby the marker solution was obtained.

Example 4: NAIP assay

(1) Method of operation

 Sample solutions containing the purified NAIP at different concentrations were diluted with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride. 50 µl of each of the diluted
25 solutions was pipetted into each hole of the plate on which the primary antibodies had been provided in the solid state prepared in Example 3 (1). The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

 Next, the biotinated anti-NAIP polyclonal antibody of Example 3 (2)
30 was diluted to the concentration of 0.5 µg/ml with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride and 1 g/l casein. 100 µl of each of the diluted solutions was pipetted into each hole of

the aforementioned plate. The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

As the final step, 100 µl of the solution of horse radish peroxidase-labeled streptoavidin prepared in Example 3 (3) was pipetted into each hole of the aforementioned plate. The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

(2) Color-developing reaction and measurement of absorbance

3,3',5,5'-tetramethylbenzidine was dissolved into N,N-dimethylformamide so that the concentration of 3,3',5,5'-tetramethylbenzidine was 50 mmol/l. The obtained solution was diluted to 1/100 with 100 mmol/l sodium acetate buffer (pH 5.5) and then filtered by a filtering paper. 0.1 ml of aqueous hydrogen peroxide (10 g/l) was added to 10 ml of the solution, whereby the color developing solution was obtained. 50 µl of the color developing solution was pipetted into each hole of the aforementioned plate. The plate was stored at 30°C for 30 minutes. Thereafter, 50 µl of sulfuric acid (2 mol/l) was pipetted into each hole of the plate, so that the reaction stopped. Absorbance was then measured at 450 nm.

(3) Results

Fig. 1 is a graph that shows the relationship between the concentration of the purified NAIP in the sample solution and the absorbance measured by the aforementioned method. The concentration of NAIP in the sample was measurable because the values thereof resided within the measurable range of 4 ng/ml to 20 ng/ml.

From the results, it was confirmed that, if the NAIP concentration is unknown for a sample, the concentration of NAIP of the sample can be accurately assayed on the basis of the absorbance thereof by utilizing, for example, the measurement results as shown in Fig. 1 as the reference line.

Example 5: Western Blot

5 (1) Preparation of sample for SDS gel electrophoresis

Mononuclear cells were separated from 10 ml of normal human peripheral blood by using Ficoll Paque PLUS (by Amasham-Pharmacia Co.). The obtained mononuclear cells were fixed by 5-10 % trichloroacetic acid and then were separated by centrifuging. The separated cells were dissolved into
10 a Tris buffer containing lithium dodesyl sulfate (2%), urea (8M), DTT (1%), and Triton X-100 (1%).

(2) Western Blot

Using the aforementioned sample carried out SDS gel electrophoresis,
15 and the result was transferred to a PVDF film. The PVDF film on which the transfer had been done was treated overnight at 4°C with TBS containing skimmed milk (10%) and Tween 20 (0.05%). The PVDF film was then washed with TBS (TBST) containing Tween 20 (0.05%). Each antibody was diluted with TBST in an appropriate manner and allowed to react at the room
20 temperature for 2 hours. Then, after washing with TBST, peroxylase-labeled anti-rabbit Ig antibody or anti-mouse Ig antibody (by Amasham-Pharmacia Co.) was added for reaction that proceeded at the room temperature for 1 hour. After washing with TBST, the treatment with the ECL PLUS reagent (by Amasham-Pharmacia Co.) and exposure onto an X-ray
25 film followed, whereby signals were obtained.

(3) Results

The results are shown in Fig. 2. In all of the three types of blots in which the monoclonal antibodies were used, signals of 160 kDa which had
30 been observed for the anti-NAIP polyclonal antibody were detected.

From the aforementioned results, it was confirmed that the monoclonal antibodies hnm365, hnm381 and hnm841 prepared in

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CLAIMS

1. An anti-NAIP monoclonal antibody recognizing a human apoptosis inhibitory protein NAIP having the amino acid sequence of SEQ ID NO: 1, which is produced from hybridoma prepared by fusing a myeloma cell line with antibody-producing cell of mammal immunized by antigen containing a polypeptide which comprises amino acid sequence of the 256-586th, the 841-1052nd or parts thereof in SEQ ID NO: 1

2. Anti-NAIP monoclonal antibody hnm365, which is produced from hybridoma 656-1 (FERM BP-6919), and its epitope is the 354-365th region in SEQ ID NO: 1.

3. Anti-NAIP monoclonal antibody hnm381, which is produced from hybridoma 656-2 (FERM BP-6920), and its epitope is the 373-387th region in SEQ ID NO: 1.

4. Anti-NAIP monoclonal antibody hnm841, which is produced from hybridoma 841 (FERM BP-6921), and its epitope is the 841-1052nd region in SEQ ID NO: 1.

5. A method of assaying NAIP, which comprises contacting a marker-labeled anti-NAIP monoclonal antibody of claim 1 with a sample containing NAIP thereby binding the marker-labeled antibody with NAIP, and measuring signal strength of the marker in the bound structure.

6. The method of assaying NAIP of claim 5, wherein the anti-NAIP monoclonal antibody is any one of the monoclonal antibodies of claims 2 to 4.

7. The method of assaying NAIP of claim 5 or 6, wherein the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

8. A method of assaying NAIP which comprises contacting an anti-NAIP primary antibody with a sample containing NAIP thereby binding the primary antibody with NAIP, further binding the bound structure with an anti-NAIP secondary antibody, and measuring signal strength of a marker bound with the secondary antibody, wherein:

(1) the primary antibody and the secondary antibody are both the anti-NAIP monoclonal antibody of claim 1;

(2) the primary antibody is the anti-NAIP monoclonal antibody of claim 1 and the secondary antibody is an anti-NAIP polyclonal antibody; or

(3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is the anti-NAIP monoclonal antibody of claim 1.

9. The method of assaying NAIP of claim 8, wherein the primary antibody is immobilized on solid phase.

10. The method of assaying NAIP of claim 8 or 9, wherein the anti-NAIP monoclonal antibody is any one of the monoclonal antibodies of claims 2 to 4.

11. The method of assaying NAIP of claim 8, 9 or 10, wherein the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

12. A NAIP assay kit at least including:

(a) a plate on which an anti-NAIP primary antibody is immobilized; and

(b) an anti-NAIP secondary antibody labeled with a marker, wherein:

(1) the primary antibody and the secondary antibody are both the anti-NAIP monoclonal antibody of claim 1;

(2) the primary antibody is the anti-NAIP monoclonal antibody of claim 1 and the secondary antibody is an anti-NAIP polyclonal antibody; or

(3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is the anti-NAIP monoclonal antibody of claim 1.

13. The NAIP assay kit of claim 12, wherein the anti-NAIP monoclonal

antibody is any one of the monoclonal antibodies of claims 2 to 4.

14. The NAIP assay kit of claim 12 or 13, wherein the marker is a radioactive isotope or a fluorescent colorant.

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15. The NAIP assaying kit of claim 12 or 13, wherein the marker is an enzyme and the kit further includes:

(c) a substrate which develops a color by the enzyme activity.

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16. A NAIP assaying kit at least including:

(a) a plate on which an anti-NAIP primary antibody is immobilized;

(b) an anti-NAIP secondary antibody; and

(c) a marker to be bound with the secondary antibody, wherein:

(1) the primary antibody and the secondary antibody are both the anti-

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NAIP monoclonal antibody of claim 1;

(2) the primary antibody is the anti-NAIP monoclonal antibody of claim 1 and the secondary antibody is an anti-NAIP polyclonal antibody; or

(3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is the anti-NAIP monoclonal antibody of claim 1.

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17. The NAIP assaying kit of claim 16, wherein the anti-NAIP monoclonal antibody is any one of the monoclonal antibodies of claims 2 to 4.

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18. The NAIP assaying kit of claim 16 or 17, wherein the marker is a radioactive isotope or a fluorescent colorant.

19. The NAIP assaying kit of claim 16 or 17, wherein the marker is an enzyme and the kit further includes:

(d) a substrate which develops a color by the enzyme activity.

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ABSTRACT

An anti-NAIP monoclonal antibody recognizing a human apoptosis inhibitory protein NAIP having the amino acid sequence of SEQ ID NO: 1, which is produced from hybridoma prepared by fusing a myeloma cell line with antibody-producing cell of mammal immunized by antigen containing a polypeptide which comprises amino acid sequence of the 256-586th, the 841-1052nd or parts thereof in SEQ ID NO: 1, NAIP assay method using the antibody, and NAIP assay kits.

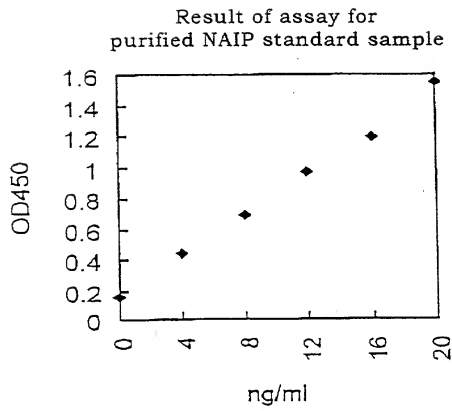
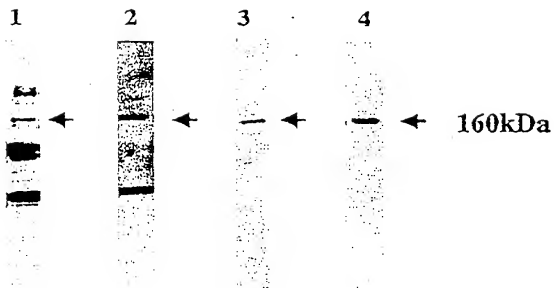
Fig. 1

Fig. 2

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute () PCT () DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: MONOCLONAL ANTIBODIES AGAINST HUMAN APOPTOSIS INHIBITORY PROTEIN NAI1 AND METHOD FOR ASSAYING THE NAI1

of which is described and claimed in:

() the attached specification, or

(X) the specification in application Serial No. _____, filed April 26, 2001, and with amendments through _____, or

() the specification in International Application No. PCT/JP99/05841, filed October 22, 1999, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Japan	1998-304550	October 26, 1998	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Warren M. Cheek, Jr., Reg. No. 33,367; Nils Pedersen, Reg. No. 33,145; Charles R. Watts, Reg. No. 33,142; and Michael S. Huppert, Reg. No. 40,268, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., as well as any other attorneys and agents associated with Customer No. 000513, to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys and agents named herein to accept and follow instructions from NISHIZAWA & ASSOCIATES as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

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Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Fifth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Sixth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
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Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor *[Signature]* Date June 28, 2001
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 2nd Inventor *[Signature]* Date June 28, 2001
 Harumi SAKAI
 3rd Inventor _____ Date _____
 4th Inventor _____ Date _____
 5th Inventor _____ Date _____
 6th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U.S. Application Serial No. _____ Filing Date April 26, 2001

Applicant Reference Number 99-F-051PCT-US/YS Atty Docket No. 2001_0515A

Title of Invention MONOCLONAL ANTIBODIES AGAINST HUMAN APOPTOSIS INHIBITORY PROTEIN NAIP AND METHOD FOR ASSAYING THE NAIP